

The influence of sphingosine-1-phosphate receptor antagonists on gentamicin-induced hair cell loss of the rat cochlea

著者	中山 雅博
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The influence of sphingosine-1-phosphate receptor antagonists on gentamicin-induced hair cell loss of the rat cochlea.

(ゲンタマイシン耳毒性における蝸牛有毛細胞障害に対するスフィンゴシン 1 リン酸受容体アンタゴニストの効果)

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筑波大学大学院博士課程人間総合科学研究科

中山 雅博

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1. Introduction

Sphingolipid metabolites, such as ceramides and sphingoid bases, have been implicated in the modulation of membrane signal transduction systems and in diverse cellular processes, such as cell proliferation, survival, migration, and angiogenesis [1,2,3,4]. We recently reported that exogenously applied sphingosine-1-phosphate (S1P) protected cochlear hair cells against gentamicin ototoxicity [5]. S1P exerts its cellular responses through a family of 5 G-protein-coupled S1P receptors (S1PRs) known as S1PR₁₋₅ [6,7,8,9]. These S1PRs are differentially expressed in various cell types. S1PR₁, S1PR₂, and S1PR₃ are widely expressed in cells and tissues, whereas S1PR₄ and S1PR₅ are expressed only in the cells of the immune and nervous systems [10]. Presence of S1PR₁₋₃ has been shown in the organ of Corti [11,12], but expression of S1PR_{4,5} has never been examined before.

Aminoglycoside antibiotics are widely used for the treatment of infectious diseases. However, the clinical usage of aminoglycosides has often been limited owing to their side effects-ototoxicity and nephrotoxicity. Aminoglycosides are well known to damage cochlear inner ear hair cells, causing sensorineural hearing loss and balance disturbance [12]. Recent

findings have demonstrated that death of cochlear hair cells was elicited by gentamicin via an apoptotic pathway, at least in part [13,14]. Particularly, evidence of the involvement of the intrinsic apoptotic pathway in gentamicin ototoxicity has been demonstrated [15].

The present study was designed to investigate the expression of S1PRs in the rat cochlea and to examine the role of S1PRs in hair cell death induced by gentamicin.

2. Materials and methods

2.1. Animals

Postnatal days 3 (P3) to 5 (P5) Sprague-Dawley rats were used. All animal procedures were carried out according to the guidelines of the Laboratory Animal Research Center of Tsukuba University.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The cochlea, organ of Corti, and spiral ganglion were dissected. Total RNA was extracted from each cell, using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using total RNA (1µg) with a GeneAmp PCR System 9600 (Perkin Elmer, Tokyo, Japan). The mRNA expression levels of S1PR₁₋₅ were detected by conventional RT-PCR with Taq polymerase (Takara, Shiga, Japan).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA integrity. S1PR primer sequences were as follows:

(1) S1PR₁-F: 5'-AGCGTTTGTCTGGAGAAGTACC-3'

S1PR₁-R: 5'-TAGCAAGGAGGCTGAAGACTGA -3'

(2) S1PR₂-F: 5'-CCTGAGAAGGTTTCAGGAACACTAC-3'

S1PR₂-R: 5'-CCCAATGAGCATCAACATTCGAC-3'

(3) S1PR₃-F:5'-ATGTCCGGTAGGAAGACGTTCA-3'

S1PR₃-R: 5'-AAGAAAGCACGCCGCATCTC-3'

(4) S1PR₄-F:5'-GATCTTGGTGGCTTTTGTGG-3'

S1PR₄-R: 5'-CTCTCGCATCTTGAAGCTGA-3'

(5) S1PR₅-F:5'-CCAGTGCACAAATGCCAA-3'

S1PR₅-R: 5'-GTTGTAGTGAAGGACGATGAC-3'

(6) GAPDH-F:5'-AAGGTCATCCCAGAGCTGAA-3'

GAPDH-R: 5'-GTTGAAGTCACAGGAGACAACC-3'

2.3. Culture technique

The basal turn of the organ of Corti was dissected and cultured according to the methods of Van de Water and Ruben [16] and Sobkowitz et al. [17]. Cochlear explants were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 25mM HEPES, and 30U/mL penicillin. They were cultured in an incubator at 37°C with 5% CO₂ at 95% humidity. Cochlear cultures were maintained in the above-described medium overnight (8-12 hours) and then exposed to a medium containing 35µM gentamicin for 48 hours to assess the effects of S1PR antagonists

[18,19]. Each S1PR antagonist was tested at concentrations of 1 to 100 μ M.

2.4. S1P receptor antagonists

S1P and (R)-3-amino-(3-hexylphenylamino)-4-(oxobutylphosphonic) acid (W146, a selective S1PR₁ antagonist) and 1-[1,3-Dimethyl-1,4-(2-methylethyl)-1H-pyrazolo[3,4b]pyridin-6-yl]-4-(3,5-dichloro-4-pyridinyl)-semicarbazide (JTE013, a selective S1PR₂ antagonist) were purchased from Sigma Japan (Tokyo, Japan). 2-undecyl-thiazolidine-4-carboxylic acid (BML241, a selective S1PR₃ antagonist) was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). W146 was initially dissolved in methanol to 10 mM and stored at -20 °C. JTE013 was initially dissolved in dimethyl sulfoxide (DMSO) to 10 mM and stored at -20°C. BML241 was initially dissolved in dimethylformamide (DMF) to 10 mM and stored at -20°C. Each antagonist was diluted in the culture medium to the final concentration immediately before use.

2.5. Cytochemistry

At the end of the tissue culture, the explants were fixed with 4%

paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized with 5% Triton X-100 (Sigma, St Louis, MO) in PBS with 10% fetal bovine serum (FBS) for 10 minutes. The specimens were stained with phalloidin with a conjugated Alexa Fluor probe (1:100, Molecular Probes, Carlsbad, CA) at room temperature for 1 hour. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and the cuticular plates of hair cells [12,18]. All experiments consisted of 8 to 20 explants per experimental group.

2.6. Assessment of cochlear hair cell damage

Hair cells were characterized as missing if no stereocilia or cuticular plates were observed by phalloidin staining. Quantitative results were obtained by evaluating 30 outer hair cells in a given microscopic field [15]. The average of 3 separate counts was used to represent each culture.

2.7. Western blot analysis

The organ of Corti and the spiral ganglion were homogenized in lysis buffer containing 0.25 M sucrose, 50 mM dithiothreitol, 3 mM HEPES (pH

7.9), 0.5 mM EGTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 mM aprotinin, 21µM leupeptin, 36 µM bestatin, 15µM pepstatin A, 14µM (4-guanidino) butane, and 1% Triton X-100. After centrifugation (x12000, 10 minutes, 4°C), the supernatants were used for Western immunoblot analysis. Appropriate volumes of the samples (10µg/lane), were mixed with equal volumes of sample buffer (100 mM Tris-HCL, PH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue), heated at 95°C for 5 minutes, and then subjected to SDS-PAGE using 10% polyacrylamide gels. The proteins were transferred by semidry electroblotting from the gels to polyvinylidene difluoride membranes for 120 minutes. The blots were then blocked with the primary antibodies, cleaved caspase 9 or caspase 3 polyclonal antibody (Cell Signaling Technology, Denvers, MA, USA) for 18 hours at 4°C. Next, the blots were incubated with an appropriate second antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology), for 1 hour. Immunoreactive bands were visualized using enhanced chemiluminescence(ECL kit; GE Healthcare Japan, Tokyo, Japan). The scanned immunoblot images were densitometrically analyzed with an ImageQuant LAS4000 mini imager (GE Healthcare Japan, Tokyo,

Japan). The result of each ratio of examined protein / β -actin protein was obtained from independent measurements (n=6 per group for each measurement).

2.8. Data analysis

All data were expressed as the means \pm SEMs. Statistical analysis was performed by unpaired *t* - tests or 1 way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests, as required (StatView 5.0). Probability values less than 0.05 were considered significant.

3. Results

3.1 Expression of S1PRs in the cochlea

Nonquantitative RT-PCR analysis clearly detected S1PR₂ mRNA in the organ of Corti and spiral ganglion. S1PR₁ and S1PR₃ mRNA was also detected in both the organ of Corti and the spiral ganglion. In contrast to the results for S1PR₁₋₃, no expression of S1PR₄ or S1PR₅ mRNA was observed both in either the organ of Corti or the spiral ganglion (Fig.1).

3.2 Effects of S1PR antagonists on cochlear hair cells (control study)

In control explants maintained in the initial medium for 48 hours without exposure to gentamicin, almost all hair cells including 1 row of inner hair cells and 3 rows of outer hair cells were present (Fig.2A). The effects of each antagonist and each solvent (W146, DMSO; JTE013, Methanol; BML241,DMF) on the outer hair cells were examined and compared with the control explants. These antagonists and their solvents did not induce any significant outer hair cell loss when explants were cultured for 48 hours in the medium containing these agents (data not shown).

3.3 Effects of S1PR antagonists on gentamicin ototoxicity

The effect of each antagonist on gentamicin-induced cochlear hair cell loss was examined using basal turn explants. The explants treated with gentamicin alone showed significantly reduced numbers of outer hair cells (Fig. 2A). Compared to the explants treated with gentamicin alone (the gentamicin control group), JTE013 increased the ratio of outer hair cell loss induced by gentamicin at the concentrations of 10 and 100 μ M (Fig. 2A and B) (Bonferroni test: $p < 0.05$). The ratio of outer hair cell loss differed significantly between the 10 and 100 μ M subgroups, suggesting the dose-dependency of outer hair cell damage within this range of concentrations (Bonferroni test: $p < 0.05$). However, W146 (1, 10, 100 μ M) or BML241 (1, 10, 100 μ M) did not influence gentamicin ototoxicity (Fig. 2A and B).

3.4. Activation of the intrinsic apoptotic pathway in gentamicin ototoxicity

The protein expression levels of cleaved caspase-3 and cleaved caspase-9 in the cochlea were examined by Western blot analysis (Figs. 3 and 4). Neither cleaved caspase 3 nor cleaved caspase 9 was detected in the normal organ of Corti cultures (without gentamicin). Gentamicin induced expression of cleaved caspases 3 and 9. In addition, JTE013 treatment significantly

increased expression levels of cleaved caspases 3 and 9 as compared with the gentamicin-alone group.

4. Discussion

Expression of S1PR₁₋₃ in the organ of Corti was shown in previous studies [10,11]. In the present study, we demonstrated that S1PR₁₋₃ mRNA was expressed in the spiral ganglion as well as in the organ of Corti of Sprague-Dawley rats'. However, S1PR₄ and S1PR₅ mRNA was not detected in the cochlea. The present findings seem reasonable because S1PR₁₋₃ are widely expressed in various tissues, whereas expression of S1PR₄ is reportedly limited to the lymphoid and hematopoietic tissues and that of S1PR₅ to the central nervous system [10,20].

On the basis of the results obtained for S1PR expression in the cochlea, we examined the effects of S1PR₁₋₃ antagonists on gentamicin ototoxicity. The results showed that the ratio of outer hair cell loss increased in the JTE013-treated group as compared with in the gentamicin-alone group. On the other hand, W146 or BML241 treatment had no effect on the gentamicin-induced damage of hair cells. S1P, produced intracellularly, generally functions as an anti-apoptotic substance. In our previous study, S1P protected hair cells against gentamicin ototoxicity [5]. The present findings strongly suggest that S1PR₂ signaling is important for cochlear hair

cell survival in gentamicin ototoxicity. Kono et al. [10] and MacLennan et al. [21] recently reported that S1P signaling was essential for the maintenance of the cochlea in infantile animals via the activation S1PR₂. The S1PR₂ -null mice were profoundly deaf, and the structure of the cochlea in S1PR₂ null mice was abnormal [10,21]. Their findings support our assumption that S1PR₂ signaling influences the fate of cochlear hair cells during gentamicin exposure.

The mechanisms of aminoglycoside-induced cochlear hair cell death are not fully known. However, it has been reported that the intrinsic apoptotic pathway is one of the major pathways leading to cochlear hair cell death [15,22]. We examined the activation of the intrinsic apoptotic pathway by measuring cleaved caspases 3 and 9. In agreement with the previous reports [15,22], gentamicin activated the intrinsic apoptotic pathway. Namely, both cleaved caspases 3 and cleaved caspases 9 were detected after gentamicin exposure. Furthermore, JTE013, an S1PR₂ antagonist, enhanced the cleavage of caspases 3 and 9 induced by gentamicin. On the basis of the present finding, it is assumed that S1P may inhibit the intrinsic apoptotic pathway via S1PR₂ in gentamicin ototoxicity.

5. Conclusions

An S1PR₂ antagonist enhanced the intrinsic apoptotic pathway of cochlear outer hair cells in gentamicin ototoxicity. This finding suggests the importance of S1PR₂ signaling in cochlear hair cell survival against ototoxic insults.

6. Acknowledgments

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Figures

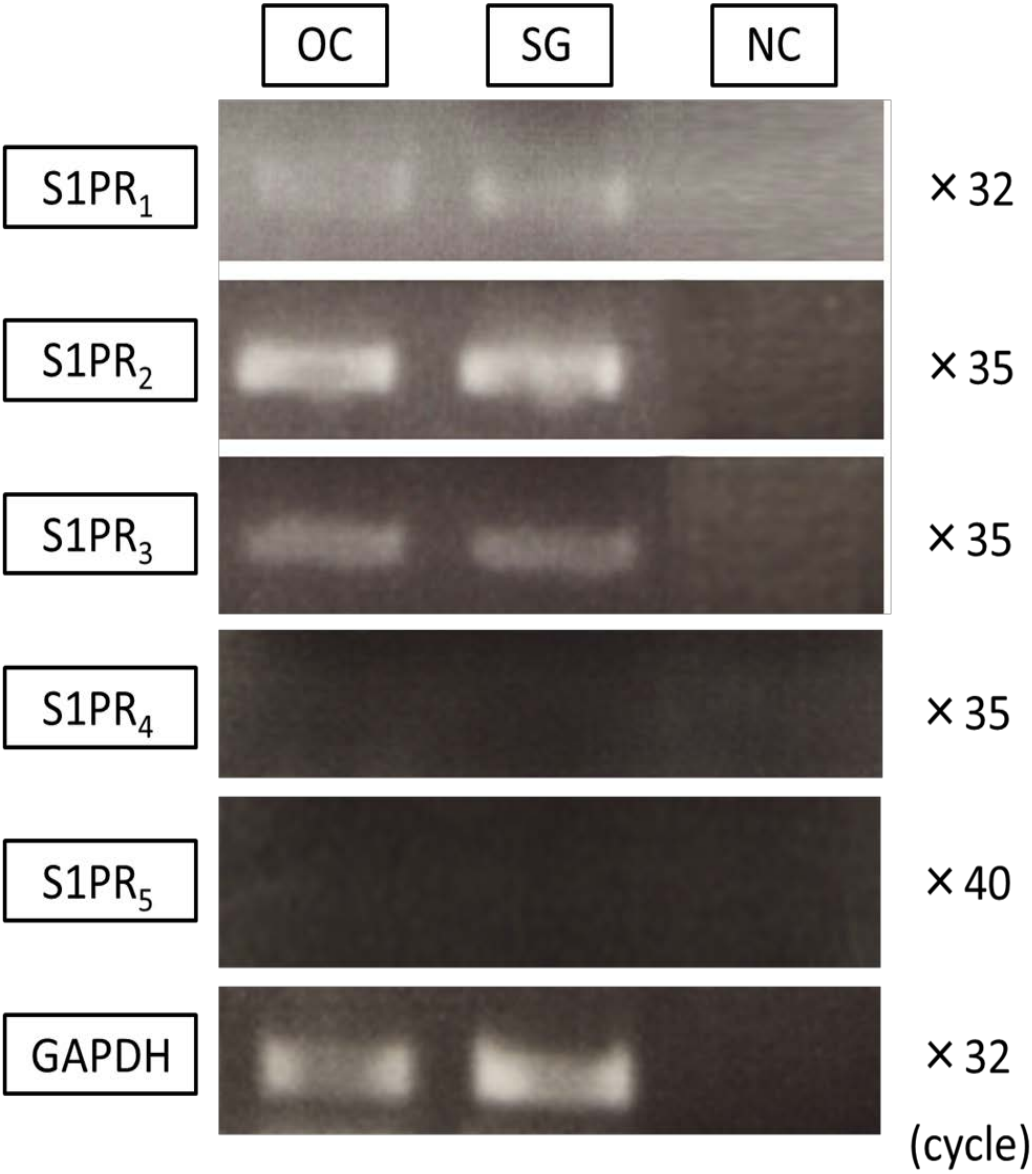


Figure 1

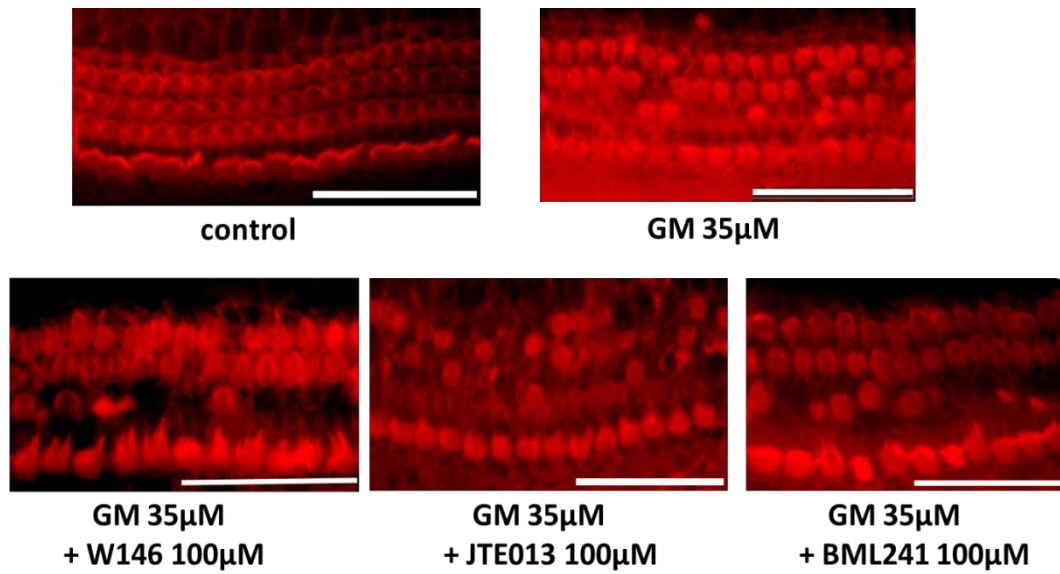


Figure 2 (A)

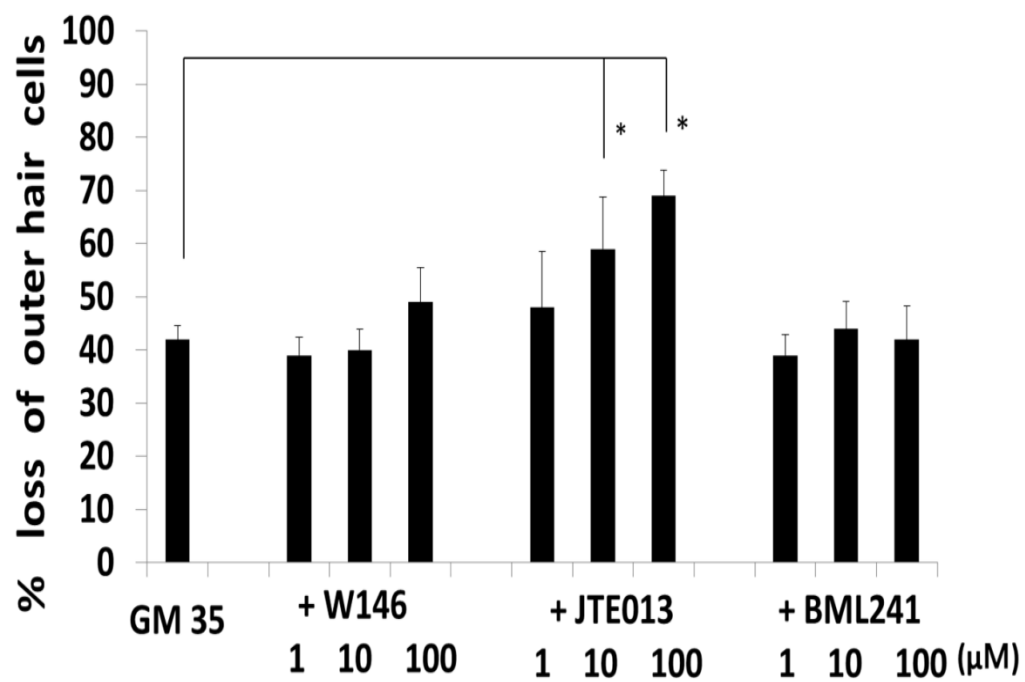


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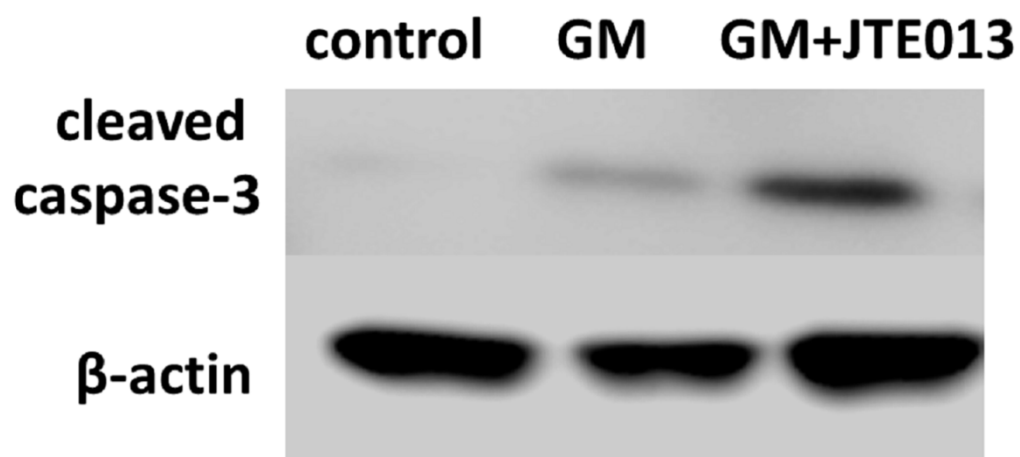


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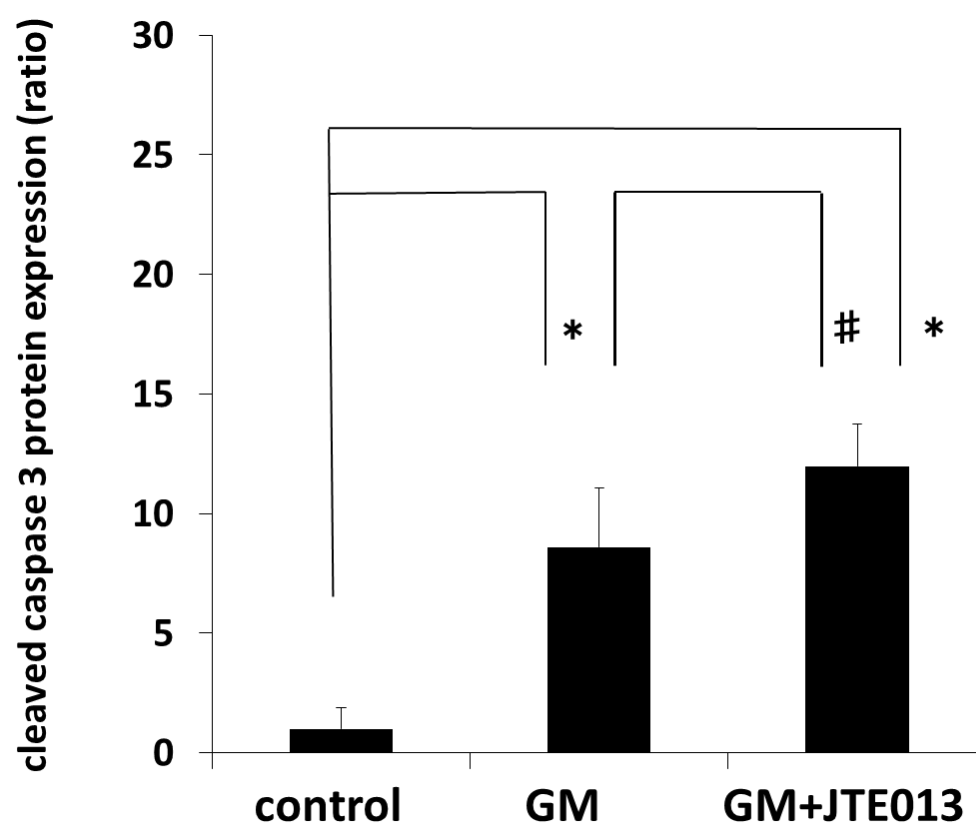




Figure 4(A)

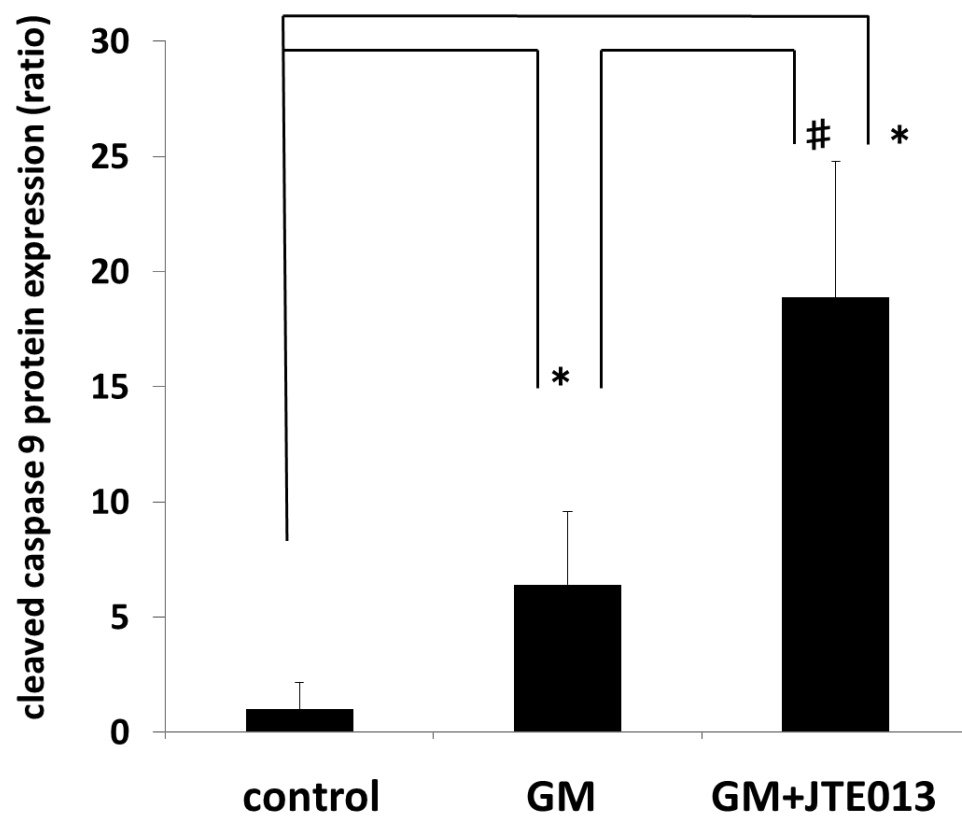


Figure Legends

Figure 1. Expression of S1PRs in the cochlea. RT-PCR analysis demonstrated that 3 S1P receptors (S1P₁₋₃) were expressed in the organ of Corti (OC) and spiral ganglion (SG) of Sprague-Dawley rats on postnatal days 3 (p3) to 5 (p5). However, the other 2 S1P receptors (S1P_{4,5}) were not detected. GAPDH primers served as a cDNA loading control. (NC; negative control)

Figure 2. Effects of S1P₁₋₃ antagonists on cochlear hair cells. The organ of Corti explant was cultured with or without gentamicin. (A) Representative microphotographs. (B) The effects of S1P antagonists on gentamicin ototoxicity were examined. The organ of Corti was cultured with 35 μ M gentamicin alone or with 35 μ M gentamicin plus 100 μ M of each S1PR antagonist for 48 hours. JTE013 (an S1PR₂ antagonist) increased hair cell loss at 10 and 100 μ M as compared with the culture with gentamicin alone ($p < 0.05$, 1-way ANOVA and Bonferroni test). Scale bar: 50 μ m.

Figure 3. Expression levels of cleaved caspase 3 in the organ of Corti were assessed by Western blot analysis (n=6 in each group). (A) Typical blot

images. β -actin was used as an internal control. Cleaved caspase 3 was not detected in the control organ of Corti (without gentamicin). Cleaved caspase 3 was detected in the explant exposed to gentamicin. JTE013 increased expression levels of cleaved caspase 3. (B) Quantitative analysis of cleaved caspase-3. Control: tissue sample from organ of Corti maintained in the initial medium for 48 h. GM: organ of Corti treated with gentamicin 35 μ M for 48 h. GM+JTE013: organ of Corti treated with 35 μ M gentamicin plus 100 μ M JTE013 for 48 h.

Figure 4. Expression levels of cleaved caspase 9 were assessed by Western blotting. (A) Typical blot images. β -actin was used as an internal control. Cleaved caspase 9 was not detected in the control organ of Corti (without gentamicin). Gentamicin induced cleaved caspase 9, and JTE further increased expression level of cleaved caspase 9.

(B) Quantitative analysis of cleaved caspase-9. control; tissue sample from the organ of Corti maintained in the initial medium for 48h. GM; the organ of Corti treated with 35 μ M gentamicin for 48h. GM+JTE013; the organ of Corti treated with 35 μ M gentamicin plus 100 μ M JTE013 for 48 hours.